

## Whole-Genome Comparison between *Photorhabdus* Strains To Identify Genomic Regions Involved in the Specificity of Nematode Interaction

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**The bacterium *Photorhabdus* establishes a highly specific association with *Heterorhabditis*, its nematode host. *Photorhabdus* strains associated with *Heterorhabditis bacteriophora* or *Heterorhabditis megidis* were compared using a *Photorhabdus* DNA microarray. We describe 31 regions belonging to the *Photorhabdus* flexible gene pool. Distribution analysis of regions among the *Photorhabdus* genus identified loci possibly involved in nematode specificity.**

*Photorhabdus* is an entomopathogenic gram-negative bacterium belonging to the *Enterobacteriaceae*. Both *Photorhabdus luminescens* and *Photorhabdus temperata* species are symbionts of entomopathogenic *Heterorhabditis* nematodes (7). Bacterial and nematode taxonomic data reveal a highly specific association between bacterial strains and nematode species. A recently described species, *Photorhabdus asymbiotica*, was never found to be associated with nematodes but was isolated from human infections (3, 12, 15, 22). Although a few studies have identified several *Photorhabdus* genes that are required for normal growth and development of the nematode (5, 6, 9, 19, 29), we have little molecular and functional data about the first step of nematode colonization and nematode specificity.

The genome sequence of *P. luminescens* subsp. *luminescens* strain TT01 revealed a high number of genes encoding proteins potentially involved in host-bacterium interaction (10). This genome also showed an impressive number of mobile or repeated genetic elements (phage remnants, IS, transposons, ERIC elements, and overrepresented families of paralogs). Furthermore, 32 genomic islands (GI) were predicted on the basis of in silico features.

The goal of this project was to identify bacterial genomic regions that are possibly involved in nematode specificity. The genomes of two strains harbored by two nematode species, *P. luminescens* subsp. *laumondii* TT01, associated with *Heterorhabditis bacteriophora*, and *P. temperata* subsp. *temperata* XINach, associated with *H. megidis*, were compared using a *Photorhabdus* TT01 DNA microarray. Since the TT01 and

XINach strains belonged to different species, genomic differences could depend on the taxonomic difference. In order to avoid this bias, the microarray comparison was also performed between TT01 and the *P. temperata* C1 strain, which was isolated from an *H. bacteriophora* nematode (20, 23). The genomic regions present in both TT01 and C1 but that were missing in XINach were considered potentially specific to strains associated with *H. bacteriophora*. *Photorhabdus* strains were stored at  $-80^{\circ}\text{C}$  and grown in Luria-Bertani broth or on 1.5% nutrient agar (Difco) at  $28^{\circ}\text{C}$ . Genomic DNA (gDNA) was extracted according to the method of Brenner et al. (8) and stored at  $4^{\circ}\text{C}$ .

The *Photorhabdus* DNA microarray used in this study is representative of 4,144 genes out of the 4,909 predicted genes of the *P. luminescens* strain TT01 chromosomal sequence (accession number NC\_005126). Paralogous genes (mainly IS and putative phages) were excluded. Primers were designed by use of a modified version of Primer 3 software (CAAT-Box [14]) to amplify specific fragments (300 to 600 bp). Probes were amplified with Titanium DNA polymerase (Clontech) from 30 ng of *P. luminescens* TT01 gDNA and purified on Multiscreen PCR filter plates (Millipore), and the probes' concentrations were adjusted to 30 ng/ $\mu\text{l}$  in 50% dimethyl sulfoxide. Quality and quantity of the final matrix were checked by gel electrophoresis of the amplified probes and sequencing of 96 randomly chosen amplified probes. Using the GenIII Amersham spotter, two replicates of each probe were spotted at different locations on glass slides (Microarray Type7 Star; Amersham). In each spotting replicate, 4,144 spots were gene probes, 100 were controls, and the remaining 364 were empty. The controls were composed of DNA from salmon sperm or *Xenorhabdus* and *Photorhabdus* genome or of housekeeping genes from *Ara-bidopsis*, rat, or *Photorhabdus*.

For the hybridization experiment, gDNA (1  $\mu\text{g}$ ) was labeled with Cy3 or Cy5 according to the Bioprime kit protocol (Invitrogen) except that the  $10\times$  deoxynucleoside triphosphate mixture was replaced by dATP, dGTP, dTTP (final, 0.12 mM; Promega), dCTP (0.06 mM; Promega), and Cy3- or Cy5-dCTP (0.02 mM; Amersham). Labeled gDNA was purified through

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TABLE 1. Regions missing from XINach described by whole-genome comparison using DNA microarray<sup>a</sup>

Locus	Gene region	Size of region in TT01 (kb)	Products of interest (similarity or function)	Matching GI <sup>b</sup>	Matching EVR, <sup>c</sup> other features
1	plu0125–plu0132	10	Unknown, Sai integrase	Part of GI plu0125–plu0169	
2	plu0136–plu0156	18	Unknown, transcriptional regulator, CoA metabolism, helicase	Part of GI plu0125–plu0169	
3	<b>plu0263–plu0269</b>	<b>8</b>	<b>Plus cluster VI (Fim-like, type 1 pili)</b>		<b>Part of EVR plu0260–plu0271 (11.7 kb, recombinase)</b>
4	<b>plu0280–plu0282</b>	<b>3</b>	<b>Phage remnant</b>		<b>Part of EVR plu0275–plu0285 (10.4 kbases, truncated, transposase, DNA ligase, phage protein)</b>
5	<b>plu0406–plu0418</b>	<b>12</b>	<b>Phage remnant and plus cluster V (mrf-like, pili mannose resistant)</b>	<b>Part of GI plu0404–plu0419</b>	
6	plu0567–plu0577	13	Sugar transport and metabolism, amino acid synthesis		Part of EVR plu0570–plu0574 (5.3 kb, ERIC sequences at 5' extremity, IS)
7	plu0597–plu0600	5	Unknown, DNA methyltransferase		EVR plu0597–plu0600 (4.7 kb, proximity of a truncated phage gene and a truncated transposase)
8	plu0752–plu0764	17	Peptide synthesis and transport, CoA metabolism	Part of GI plu0751–plu0798	
9	plu0895–plu0899	16	Cro/CI transcriptional regulator, antibiotic synthesis	Part of GI plu0884–plu0901	
10	plu0960–plu0965	27	Insecticidal toxins (loci <i>ted</i> and <i>tec</i> )	Part of GI plu 0958–plu1166	
11	plu1002–plu1005	4	Deshydratase, dioxygenase, cyanate, and benzoate transport	Part of GI plu 0958–plu1166	
12	<b>plu1207–plu1213</b>	<b>14</b>	<b>Antibiotic synthesis</b>	<b>Part of GI plu1203–plu1238</b>	
13	plu1436–plu1443	11	Antibiotic synthesis		Part of EVR plu1434–plu1448 (15.4 kb)
14	plu2727–plu2729	3	Enterobactin synthetase (entABE)		EVR plu2727–plu2729 (3.1 kb, low GC %, flanked by repeats)
15	<b>plu2792–plu2799</b>	<b>10</b>	<b>Antibiotic synthesis</b>		<b>Part of EVR plu2787–plu2800 (18.3 kb, repeat-containing proteins)</b>
16	plu3135–plu3139	7	Citrate synthase, efflux transporter, and unknown	Part of GI plu3111–plu3140	
17	<b>plu3144–plu3146</b>	<b>5</b>	<b>Isr (luxS synthesis regulated) operon, AI-2 import</b>		<b>Proximity of transposases, ERIC sequence, plu3111–plu3140</b>
18	plu3398–plu3405	6	Phage remnant, unknown proteins	Part of GI plu3379–plu3538	
19	plu3537–plu3539	5	Aminotransferase, propionate metabolism	Overlaps the right border of GI plu3379–plu3538	
20	<b>plu3724–plu3726</b>	<b>4</b>	<b>Aminobenzoyl-glutamate uptake and utilization</b>		<b>Flanks the GI plu3685–plu3723</b>
21	plu4077–plu4081	5	Truncated aldolase, deshydrogenase, transferase, unknown proteins		Part of EVR plu4075–plu4084 (12.3 kb, transposases)
22	plu4143–plu4160	19	ABC transporter, amino acid metabolism, unknown	Part of GI plu4141–plu4246	
23	plu4205–plu4219	16	Transposase, unknown proteins	Part of GI plu4141–plu4246	
24	plu4266–plu4269	5	Amino acid metabolism, ABC transporter		Part of EVR plu4254–plu4310 (61.3 kb, transposase, Rhs family protein, low GC %)
25	plu4324–plu4328	7	Unknown proteins		Part of EVR plu4318–plu4331 (16.8 kb, phage proteins, truncated integrase)
26	<b>plu4336–plu4348</b>	<b>14</b>	<b>Carotenoid biosynthesis, unknown proteins</b>		<b>Part of EVR plu4334–plu4348 (16.4 kb, transposase NTPase, C-terminal region of group II intron-associated maturase)</b>
27	plu4589–plu4591	3	Unknown, transcription regulator LysR		Part of EVR plu4587–plu4594 (5.7 kb, tRNA-Gly site insertion at 3' extremity)
28	plu4621–plu4630	15	Ferric enterobactin biosynthesis and uptake		EVR plu4621–plu4630 (14.7 kb, ATP-dependent DNA helicase RecQ at the 5' border)
29	plu4810–plu4823	15	Lipopolysaccharide biosynthesis		Part of EVR plu4796–plu4833 (38.6 kb, transposase, low GC% by place)
30	plu4873–plu4889	16	Formate metabolism, O-methyltransferase, reverse transcriptase, macrolide-efflux protein, sugar kinase		Overlaps a part of EVR plu4872–plu4884 (11.1 kb, transposase, phage proteins, low GC% by place)
31	plu4892–plu4895	6	O-methyltransferase, transposase		Part of EVR plu4890–plu4895 (9.0 kb, transposase, low GC%)

<sup>a</sup> Bold type shows loci that are present in *P. temperata* C1 and that are consequently possibly involved in specific interaction with *H. bacteriophora*.

<sup>b</sup> Genomic islands described in reference 10.

<sup>c</sup> EVR shows synteny rupture relative to the *Enterobacteriaceae* core genome (<http://www.genoscope.cns.fr/agg/mage/wwwpkgdb/>).

TABLE 2. *Photorhabdus* strains used in this study

Strain	Nematode host	Geographical origin	Reference or source
<i>Photorhabdus luminescens</i> subsp. <i>luminescens</i> Hb <sup>T</sup> FRG26	<i>Heterorhabditis bacteriophora</i> Brecon <i>Heterorhabditis</i> sp.	South Australia Guadeloupe	24 H. Mauléon
<i>Photorhabdus luminescens</i> subsp. <i>akhurstii</i> FRG04 <sup>T</sup> JM12	<i>Heterorhabditis indica</i> <i>Heterorhabditis indica</i>	Guadeloupe Jamaica	13 13
<i>Photorhabdus luminescens</i> subsp. <i>laumondii</i> TT01 <sup>T</sup> HP88	<i>Heterorhabditis bacteriophora</i> <i>Heterorhabditis bacteriophora</i> HP88	Trinidad and Tobago USA <sup>a</sup> (Utah)	13 4
<i>Photorhabdus temperata</i> C1 = NC19 K122	<i>Heterorhabditis bacteriophora</i> NC1 <i>Heterorhabditis megidis</i>	USA (North Carolina) Ireland	1 16
<i>Photorhabdus temperata</i> subsp. <i>temperata</i> XINach <sup>T</sup> HL81	<i>Heterorhabditis megidis</i> <i>Heterorhabditis megidis</i>	Russia The Netherlands	2 26
<i>Photorhabdus asymbiotica</i> subsp. <i>asymbiotica</i> US3265-86 <sup>T</sup> US3105-77	Clinical specimen Clinical specimen	USA USA	12 12
<i>Photorhabdus asymbiotica</i> subsp. <i>australis</i> AU9802892 <sup>T</sup> AU9802397	Clinical specimen Clinical specimen	Australia Australia	22 22

<sup>a</sup> USA, United States of America.

QiaQuick minicolumns (QIAGEN) according to the nucleotide removal protocol except that the wash with PE buffer was performed three times. Cy5- and Cy3-labeled genomic DNAs were mixed, vacuum dried, and resuspended with 240 µl of

hybridization buffer (30% formamide, 5× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 0.1% sodium dodecyl sulfate [SDS]). Microarray slides were prehybridized for 1 h at 42°C in the saturation buffer (5× SSC, 0.1% bovine serum albumin, 0.1% SDS), rinsed under a continuous flow of deionized water, and, after a rapid isopropanol bath, blow dried using compressed gas. Hybridization was performed at 42°C for 12 h in an Amersham automated slide processor chamber. The microarrays were washed for 10 min in water, for 4 min in 1× SSC, 0.2% SDS, and four additional minutes in 0.1× SSC, 0.2% SDS. Microarray slides were briefly washed in isopropanol, air dried, and scanned for fluorescence intensity by using an Amersham scanner.

One microarray comparison (TT01 versus XINach or TT01 versus C1) included four slides with two dye-flipped replicates. Each slide contained two spotting replicates that were scanned in two sets of measurements. Therefore, we treated the eight sets of measurements as separate slides. The signal intensity of each spot in the microarray was quantified by using Arrayvision software (Amersham). Subsequent analysis was conducted by using Microsoft Excel software. Global normalization (using the global median) was applied on the data without background correction (11, 21). Since the different controls used on each slide demonstrate good quality for spotting and hybridization, we did not use statistical analysis. For each open reading frame tested, the median from the eight normalized values was calculated and used for determining Xlnach/TT01 or CI/TT01 ratios.

Ratios from 7.61 to 0.29 were obtained. In order to determine the ratio threshold that indicates the TT01 gene was missing in XINach or C1, we selected regions of the XINach or C1 strain, amplified them with the Hercules enhanced DNA

TABLE 3. PCR assays for loci 5, 15, 17, and 26 in 14 *Photorhabdus* strains

Host and bacterial strain	Amplicon size (kb)			
	Locus 5 pilus module	Locus 15	Locus 17	Locus 26
Host: <i>Heterorhabditis bacteriophora</i>				
TT01 <sup>T</sup>	8.5	14	5.2	18
Hb <sup>T</sup>	0.5	7.4	5.2	NA
HP88	NA <sup>a</sup>	14	5.2	17
C1	NA	NA	5.2	NA
Host: <i>Heterorhabditis indica</i>				
FRG04 <sup>T</sup>	0.5	6.9	5.2	9.4
JM12	0.5	6.9	5.2	NA
Host: <i>Heterorhabditis</i> sp.				
FRG26	0.5	6.9	5.2	10
Host: <i>Heterorhabditis megidis</i>				
K122	NA	NA	0.8	NA
XINach <sup>T</sup>	NA	NA	0.8	NA
HL81	NA	NA	0.8	NA
Clinical strains				
US3265-86 <sup>T</sup>	0.6	3	1.1	1.8
US3105-77	0.6	3	1.1	1.8
AU9802892 <sup>T</sup>	NA	2.6	0.9	0.5
AU9802397	NA	2.6	0.9	0.5

<sup>a</sup> NA, not amplified.

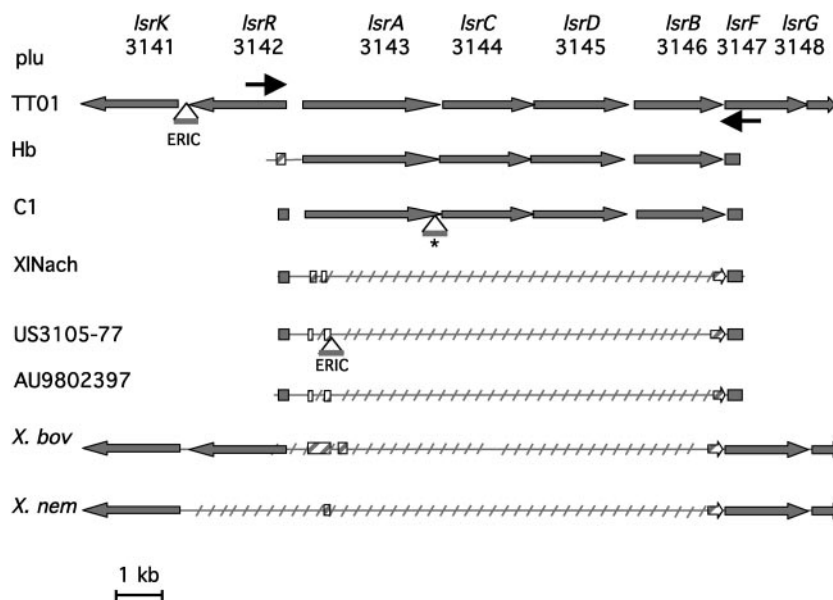


FIG. 1. Schematic representation of the deletions in the *lsr* region of several *Phototrabdus* and *Xenorhabdus* strains. Horizontal arrows represent primers designed for the long-range PCR analysis of the locus and for sequencing. Gray and hatched arrows or boxes symbolize, respectively, open reading frames and their remnants. Insertion of ERIC elements and a 27-nucleotide region (\*) are represented.

polymerase (Stratagene), and sequenced them at MilleGen (Toulouse, France). Then, 24 genes with ratios ranging between 0.4 and 1.5 were randomly selected. When ratios were equal to or smaller than 0.6, genes had less than 20% identity with the probe spotted on the microarray. When ratios were equal to or higher than 0.98, genes had more than 70% identity with the probe spotted on the microarray. For ratios between 0.7 and 0.97, the identity percentage was variable. Therefore, we fixed the ratio threshold for missing genes to 0.6. According to this criterion, 449 (10.5%) and 357 (8.5%) of the TT01 genes present on the DNA microarray were missing in XINach and C1, respectively.

In the XINach strain, no large regions, such as canonic genomic islands, were absent relative to the reference strain TT01. Then, we searched for regions containing at least three contiguous genes missing from the XINach genome that represent at least 50% of the TT01 genomic region. Thirty-one regions missing from XINach were identified (Table 1). Genes contained in these regions mainly belong to the following putative functional classes: phage remnants, pilus biosynthesis, antibiotic biosynthesis, insecticidal toxins, iron uptake, and amino acid metabolism. Among the 31 regions missing from XINach, only 8 were present in C1 (bold type in Table 1). Therefore, these eight regions may potentially be involved in *H. bacteriophora* interaction. Note that our DNA microarray analysis led to the identification of part of the flexible gene pool of *Phototrabdus* strains (17). Indeed, 29 of the regions missing from XINach fit with in silico predicted mobile regions. Thirteen regions were or belonged to previously described GIs (10). Furthermore, using the Microbial Genome Annotation System (<http://www.genoscope.cns.fr/agc/mage/wwwpkgdb/>), 16 regions matched with enterobacterial variable regions (EVRs). The EVRs were gene blocks that were inserted at the location of synteny rupture in the enterobacterial core ge-

nome. Their sizes (3 to 62 kb) and their rich content in mobile elements evoked the *Yersinia* "difference regions," which belong to the intra- and interspecific *Yersinia* flexible gene pool (18, 25).

Since nematodes are not able to grow on lawns of clinical strains (12), we assume that regions involved in nematode colonization are absent from clinical strain genomes. *P. asymbiotica* US3105-77, a clinical strain, is being sequenced ([http://www.sanger.ac.uk/Projects/P\\_asymbiotica/](http://www.sanger.ac.uk/Projects/P_asymbiotica/)). Using the BLASTP algorithm available from this site, we examined the presence of the eight regions possibly involved in *H. bacteriophora* interaction. Loci 3, 4, and 12 and the putative phage module of locus 5 were present in the clinical strain. Therefore, these loci are likely not involved in bacterial interaction with *H. bacteriophora*.

To further test the correlation between the TT01- and C1-specific regions and the interaction with *H. bacteriophora*, we studied the distribution of the putative pilus module of locus 5 (matching with a previously described GI), loci 15 and 26 (matching with EVRs), and locus 17 (not matching with any GI or EVR) in 14 *Phototrabdus* strains representative of the genus (Table 2) by PCR amplification. Primers were designed in flanking borders of the loci (primer sequences can be sent to readers upon request). gDNAs were amplified with the Hercules enhanced DNA polymerase (Stratagene). PCR products were analyzed by electrophoresis in 0.5% agarose gels (Table 3). When amplification succeeded, the PCR fragments were of various sizes. The amplification sizes of loci 5, 15, and 26 were not clearly correlated with the nematode host species. By contrast, locus 6 had a homogeneous size (5.2 kb) in bacteria carried by *H. bacteriophora*, *Heterorhabditis indica*, or a *Heterorhabditis* sp., whereas the fragment sizes were smaller than 1.1 kb in strains harbored by *H. megidis* or clinical strains. It is noteworthy that, except for locus 6, *P. temperata* gDNA



was never amplified, suggesting a divergent core genome in this species.

Locus 6 is similar to the *Salmonella enterica* serovar Typhimurium and *Escherichia coli* *lsr* region, which encodes an inner ABC transporter and a cytoplasmic phosphorylation-processing system of the autoinducer AI-2, involved in quorum sensing (27, 28, 30). In order to check the previous data, PCR products of locus 6 were purified by using the Montage PCR kit (Millipore) and sequenced with PCR primers at MilleGen (Toulouse, France). We also added for comparison the *lsr*-like locus of *Xenorhabdus bovienii* and *Xenorhabdus nematophila* ATCC 19061 (<http://xenorhabdus.danforthcenter.org/>), a genus closely related to *Photorhabdus* (7). Multiple alignments were performed with the ClustalW program (<http://searchlauncher.bcm.tmc.edu/multi-align/multi-align.html>). The *lsr* locus was similar in TT01, Hb, and C1, three bacterial strain carried by *H. bacteriophora* (Fig. 1). In the US3105-77, AU9802397, and XINach strains and in *Xenorhabdus* strains, the *lsrA*, *lsrD*, *lsrC*, and *lsrB* genes were missing. Furthermore, various *lsrA*, *lsrB*, and *lsrR* remnants were observed, showing that the *lsr* locus underwent independent deletions in these latter strains. Therefore, the *lsr* locus is an ancestral locus in the *Photorhabdus* and *Xenorhabdus* strains. The bacterial association with *H. bacteriophora* is possibly a selective pressure for the conservation of the *lsr* locus, whereas association with other nematode hosts leads to *lsr* locus loss by genomic decay.

Taken together, these data suggest that the *lsr* locus is possibly involved in the specific interaction with *H. bacteriophora*. In *S. enterica* serovar Typhimurium and *E. coli*, it was suggested that the Lsr transporter has a role in removing the AI-2 signal from the external environment in order to terminate cell-cell signaling (27, 28, 30). In nematode interaction, the termination of cell-cell signaling could be an important signal that allows a bacterial physiological shift, for example, in the insect cadaver, when bacteria recolonize the nematode intestinal tract.

In summary, this work shows that the DNA microarray is a powerful tool for selecting some genes or genomic regions potentially involved in bacterium-host interaction. Additionally, this study allowed the identification of a part of the flexible gene pool within the *Photorhabdus* genus.

**Nucleotide sequences accession numbers.** Sequences of PCR products of *Photorhabdus* locus 6 have been deposited in EMBL under accession numbers AJ967010, AM039953, AJ966980, AJ966979, and AJ967009.

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